Clinical Scale Zinc Finger Nuclease-mediated Gene Editing of PD-1 in Tumor Infiltrating Lymphocytes for the Treatment of Metastatic Melanoma

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Programmed cell death-1 (PD-1) is expressed on activated T cells and represents an attractive target for gene-editing of tumor targeted T cells prior to adoptive cell transfer (ACT). We used zinc finger nucleases (ZFNs) directed against the gene encoding human PD-1 (PDCD-1) to gene-edit melanoma tumor infiltrating lymphocytes (TIL). We show that our clinical scale TIL production process yielded efficient modification of the PD-1 gene locus, with an average modification frequency of 74.8% (n = 3, range 69.9–84.1%) of the alleles in a bulk TIL population, which resulted in a 76% reduction in PD-1 surface-expression. Forty to 48% of PD-1 gene-edited cells had biallelic PD-1 modification. Importantly, the PD-1 gene-edited TIL product showed improved *in vitro* effector function and a significantly increased polyfunctional cytokine profile (TNFα, GM-CSF, and IFNγ) compared to unmodified TIL in two of the three donors tested. In addition, all donor cells displayed an effector memory phenotype and expanded approximately 500–2,000-fold *in vitro*. Thus, further study to determine the efficiency and safety of adoptive cell transfer using PD-1 gene-edited TIL for the treatment of metastatic melanoma is warranted.

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INTRODUCTION

The ability to harness the immune system to induce tumor regression has revolutionized the treatment of patients with metastatic melanoma.[1–4] Administration of high-dose interleukin-2 (IL-2) or checkpoint modulation can induce tumor regression in these patients.[2–4] In addition, adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) offers an effective therapy for metastatic melanoma with an overall response rate of 49–72% with approximately 20% of patients achieving a durable complete regression.[5] Given this success, extensive efforts are underway to develop more effective TIL therapies, designed to increase the response rate and durability of responses.

A promising area of research involves targeting immune checkpoints, which are inhibitory receptors on immune cells that result in inhibition of key effector functions (activation, proliferation, cytokine release, cytoxicity) when they interact with their inhibitory ligand. One of the most studied targets for the induction of checkpoint blockade is Programmed Cell Death-1(PD-1), a member of the CD28 super family of T-cell regulators.[6–9] Its ligands, PD-L1 and PD-L2 are expressed on a variety of tumor cells, including melanoma. The interaction of PD-1 with its ligand PD-L1 inhibits T-cell effector function, results in T-cell exhaustion in the setting of chronic stimulation, and induces T-cell apoptosis in the tumor microenvironment.[7,8,10] The significance of this interaction has been demonstrated clinically in a growing number of prospective trials where either an infusion of anti-PD-1 or anti-PD-L1 monoclonal antibody was used to induce tumor regression in approximately 30–40% of patients.[11–13] However, the durability of the observed responses has yet to be determined.

Consistent with the early clinical success with PD-1 inhibition, we have previously shown that TIL isolated from metastatic melanoma express the PD-1 inhibitory receptor and have impaired effector cytokine production.[14] Indeed, the tumor reactive fraction of the TIL population is exclusively found within the PD-1 expressing subset.[15] Together, these data suggest that PD-1 inhibition may represent a critical checkpoint in the ability of metastatic melanoma to evade T-cell-mediated elimination. Thus, the ability to permanently silence PD-1 in TIL manipulated *ex vivo*, prior to ACT, represents an attractive approach to improving the efficacy of TIL, while avoiding the toxicities associated with the long-term administration of a systemic anti-PD-1 antibody.

Multiple technology platforms for research grade gene editing have been developed over the last 10 years including transcription activator-like effector nucleases, the bacterial CRISPR/Cas9 system, and zinc finger nucleases (ZFNs).[16–20] Each relies on the recognition of a specific DNA sequence within the genome to target a nuclease domain to this location and mediate the generation of a double-strand break at the target sequence. The repair of this break can result in the introduction of insertion/deletion mutations

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(indels) by the nonhomologous end-joining pathway (review[21]). If the nuclease target site resides in the coding sequence, such indels can result in the permanent disruption of the target gene product. While each technology differs with respect to the mechanism used to site-specifically guide the nuclease, all the platforms face a similar challenge for therapeutic use; namely, achieving at clinical scale the cleavage efficiency necessary for biologic impact in the primary human cell/tissue of choice while maintaining sufficient target specificity. To date, only the ZFN platform has been used to treat patients in a clinical trial using ZFNs targeting the HIV coreceptor, CCR5, for the clinical scale production of autologous CD4 T cells refractory to HIV infection for ACT in subjects with HIV.[22,23] The results of the initial phase 1 clinical trial showed that the treatment was generally safe and well tolerated, resulted in increased CD4 T-cell levels in all subjects and demonstrated protection of the modified T cells during an antiretroviral drug treatment interruption.[23] The implications for this technology to improve patient therapies are numerous, and the ability to harness

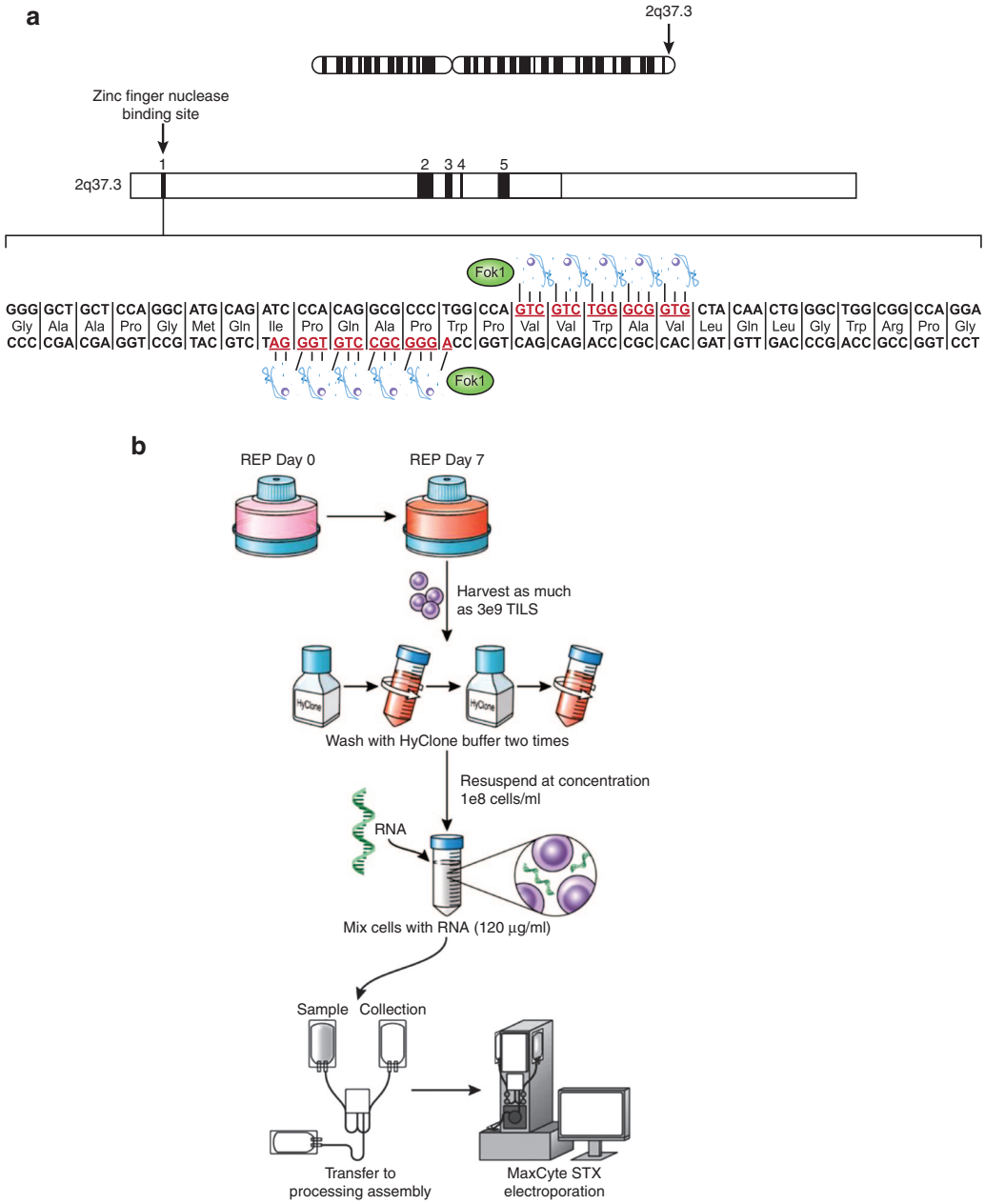

Figure 1 Schematic representation of the modified tumor infiltrating lymphocytes (TIL) process and zinc finger nuclease (ZFN) target site at the PD-1 locus. (**a**) Schematic of the ZFN target site within the PD-1 gene at the 2q37.3 location. Each ZFN comprises five zinc finger domains that recognize a composite 15bp target site (in red). Each ZFP is coupled to an obligate heterodimer mutant of the FokI endonuclease that mediates dimerization dependent DNA cleavage.[46] (**b**) Work-flow of large clinical scale RNA electroporation with PD-1 targeted ZFN. TIL were induced to rapidly expand (rapid expansion protocol (REP) day 0) and cultured in a G-Rex100 flask at 37 °C and 5% CO_2. On day 7 of the REP, TIL were harvested and washed two times with Hyclone Electroporation Buffer. Cells were then counted and resuspended in electroporation buffer at a concentration of 1×10^8/ml. Cells were mixed with 120 µg/ml of PD-1 ZFN RNA and transferred to the CL-2 processing assembly for electroporation on the Maxcyte GT Flow Transfection System.

targeted genome editing in the setting of ACT for the treatment of cancer has yet to be fully explored.

Given the success of ongoing clinical trials using inhibitory antibodies against PD-1, our aim was to determine the feasibility of permanently disrupting the PD-1 gene in human TIL using ZFN-mediated gene editing. Efforts were undertaken to gene edit TIL at clinical scale using flow electroporation with mRNA encoding the PD-1 specific ZFNs. Here we describe the ability of this platform to efficiently and specifically eliminate PD-1 expression within a TIL population at the scale necessary for patient treatment. As a further rationale for exploring this technology in a clinical trial, we characterized the phenotype of the modified TIL and described the effect of PD-1 gene editing on TIL proliferation and effector function.

RESULTS

Efficiency of clinical scale PD-1 gene modification

TIL were harvested from patients with metastatic melanoma as previously described, and induced to rapidly proliferate using a rapid expansion protocol (REP).[24] In three separate experiments, TIL from three donors underwent expansion (to a total of approximately 2×10^9 for each donor on day 7 of REP expansion) prior to electroporation with PD-1 ZFN encoding mRNA using MaxCyte's CL-2 flow electroporation processing assembly. At day 7 of the REP, TIL from each donor was resuspended in electroporation buffer to a final concentration of 1×10^8 cells/ml and mixed with PD-1 ZFN RNA at 120 µg/ml (**Figure 1b**). TIL were then electroporated with RNA encoding a pair of ZFNs targeting the PD-1 gene locus (**Figure 1a**). The percent indels, or percentage of mutated alleles, was determined by means of the Cel-1 assay. No difference in electroporation efficiency was observed when using either a single small-scale 3 ml processing assembly or the large-scale CL-2 assembly. ZFN-mediated gene editing occurred in 50.9% of alleles when TIL from Donor 1 were electroporated using a small scale cuvette and 47.1% when using the flow electroporation processing assembly. Similarly, gene editing occurred in 44.6% of alleles for Donor 2 and 42.2% for Donor 3 using the CL-2 assembly (**Figure 2**). Because the Cel-1 nuclease relies on the formation of heteroduplexes of reannealed modified and unmodified alleles during PCR, accuracy is reduced when the efficiency of gene editing increases beyond 50% indels, as modified sequences may reanneal to each other resulting in decreased heteroduplex formation. In order to more accurately determine genetic modification of the intended target site, genomic DNA from the same sample used in the Cel-1 assay was deep-sequenced using the Illumina platform technology (**Table 1**). The mean % indels was 74.8% (range 69.9–84.1%) for the three donors tested.

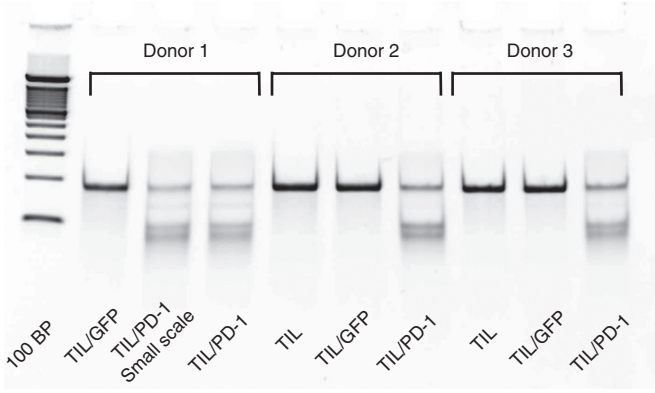

Figure 2 Efficiency of PD-1 gene disruption following electroporation with mRNA encoding PD-1-targeted zinc finger nucleases (ZFNs). Cel-1 surveyor nuclease assay performed 3 days following electroporation. ZFN-dependent gene editing occurred in 46.0% of alleles when tumor infiltrating lymphocytes from Donor 1 were electroporated using a smaller scale cuvette and 42.0% when electroporated using the flow electroporation processing assembly. Gene editing occurred in 37.0% of alleles in Donor 2 and 37.0% in Donor 3.

In order to determine the relative frequencies of mono- and bi-allelic target modification, single cell clones were generated from the bulk ZFN modified TIL population. A total of 40 clones from Donor 1 and 40 clones from Donor 3 were successfully isolated, expanded, and sequenced across the PD-1 ZFN target site. The rate of allelic modification across all clones isolated from Donor 1 was 69% (n = 55/80 alleles). Thirty-six of 40 clones from Donor 1 had insertions or deletions at the PD-1 gene locus; with a biallelic modification rate of 48% (n = 19/40 clones). For Donor 3, the rate of allelic modification across all clones isolated was 63% (n = 50/80 alleles). Thirty-four clones from Donor 3 had indels at the PD-1 locus; the percent of these clones with a biallelic modification was 40% (n = 16/40). Taken together, biallelic gene editing occurred in 44% of gene modified TIL (data not shown).

Expression of PD-1 is reduced following zinc finger nuclease-mediated gene editing

To determine the expression of PD-1 in TIL following gene editing, 7 days after electroporation (day 14 of the REP), a portion of TIL were restimulated with anti-CD3/CD28 beads for 48 hours. Both restimulated and unstimulated (no bead restimulation) TIL were assessed for PD-1 surface expression by fluorescence-activated cell sorting analysis (FACS). **Figure 3a** shows the surface expression of PD-1 on Donor 3 TIL, and is representative of all three donors. For Donor 3, 18 and 16% of TIL and TIL/GFP bulk populations expressed PD-1 on the cell surface, which was reduced to 4% following gene editing, a decrease of nearly 80%. This trend was maintained following anti-CD3/CD28 bead stimulation which increased the fraction of PD-1-positive cells in untreated or mock-electroporated TIL/GFP to 90 and 80%, respectively, while the PD-1 ZFN-treated group achieved only 31.7% PD-1$^+$ TIL. In combining all three experiments, there was a statistically significant decrease in the number of cells expressing PD-1 in the ZFN-treated TIL compared to the untransfected TIL and GFP transduced TIL (**Figure 3b**). The mean

Table 1 Quantification of PD-1 gene modification by deep sequencing using the Illumina MiSeq platform technology

	% Indels[a]		
	TIL	TIL/GFP	TIL/PD-1
Donor 1	0	0.1	84.1
Donor 2	0.1	0.6	69.9
Donor 3	0.9	0.1	70.4

[a] Percent gene modification is calculated by dividiing the indel containing sequence count by the total sequence count. The assay background level is <1% (i.e. TIL and TIL/GFP samples). The mean % PD-1 gene modification was 74.8% (range 69.9 - 84.1%) for the three donors.
TIL, tumor infiltrating lymphocytes.

number of CD3⁺ TIL expressing PD-1 was reduced by 76% on average following ZFN editing (5.6 versus 23.9% in TIL ($P = 0.015$) and 23.0% in TIL/GFP ($P = 0.027$)). A similar trend was observed in the bead-stimulated TIL ($P = 0.066$ and 0.059) (**Figure 3b**). To further characterize these differences, we performed RT-PCR on the TIL/GFP and TIL/PD-1 KO at sequential time points after anti-CD3/CD28 bead stimulation (**Figure 3c**). RNA expression of PD-1 in TIL/GFP peaked at 3 hours and subsequently declined, whereas the basal level and the activation-induced upregulation of PD-1 RNA was significantly reduced in PD-1 gene-edited TIL. Taken together, these data demonstrate that the disruption of the PD-1 locus using ZFN in TIL led to frameshift knockouts in a large portion of treated cells (with a 40–48% of biallelic knockout), which in turn significantly reduced expression of both PD-1 mRNA and protein in the ZFN-treated TIL population.

Effect of electroporation and gene editing on T-cell proliferation, phenotype, and effector function

Next we sought to determine what effect the gene editing had on T-cell proliferation, phenotype and effector function. Both TIL/GFP (mock) and TIL/PD-1 showed a modest decrease in cell proliferation during the initial REP (REP1) when rapid expansion was interrupted by the electroporation process (**Figure 4a**). However, a second rapid expansion revealed no consistent differences between TIL, TIL/GFP, and TIL/PD-1 KO with respect to proliferative capacity following REP2 (**Figure 4b**).

The phenotype of the gene-edited TIL was assessed by FACS staining using anti-CD45RA and anti-CD62L antibodies to determine the fractions of naive (CD45RA⁺/CD62L⁺), central memory (CD45RA⁻/CD62L⁺), effector memory (CD45RA⁻/CD62L⁻) and EMRA (CD45RA⁺/CD62L⁻) T cell subsets. Although slight differences in the distribution of T-cell subsets were observed across TIL from different subjects (*i.e.*, donor variability), the gene-edited TIL displayed a similar phenotype compared with unmodified cells and GFP transfected TIL. In all cases, the majority of the cells had an effector memory-like phenotype (CD45RA⁻/CD62L⁻) with slight differences in the number of cells expressing higher levels of CD45RA (**Figure 5**).

Tumor-specific effector function of TIL was evaluated by coculturing the TIL with tumor target cells and measuring cytokine release (**Figure 6**) or cytolytic activity against HLA-matched antigen-positive target cells. Tumor target cell lines used in our

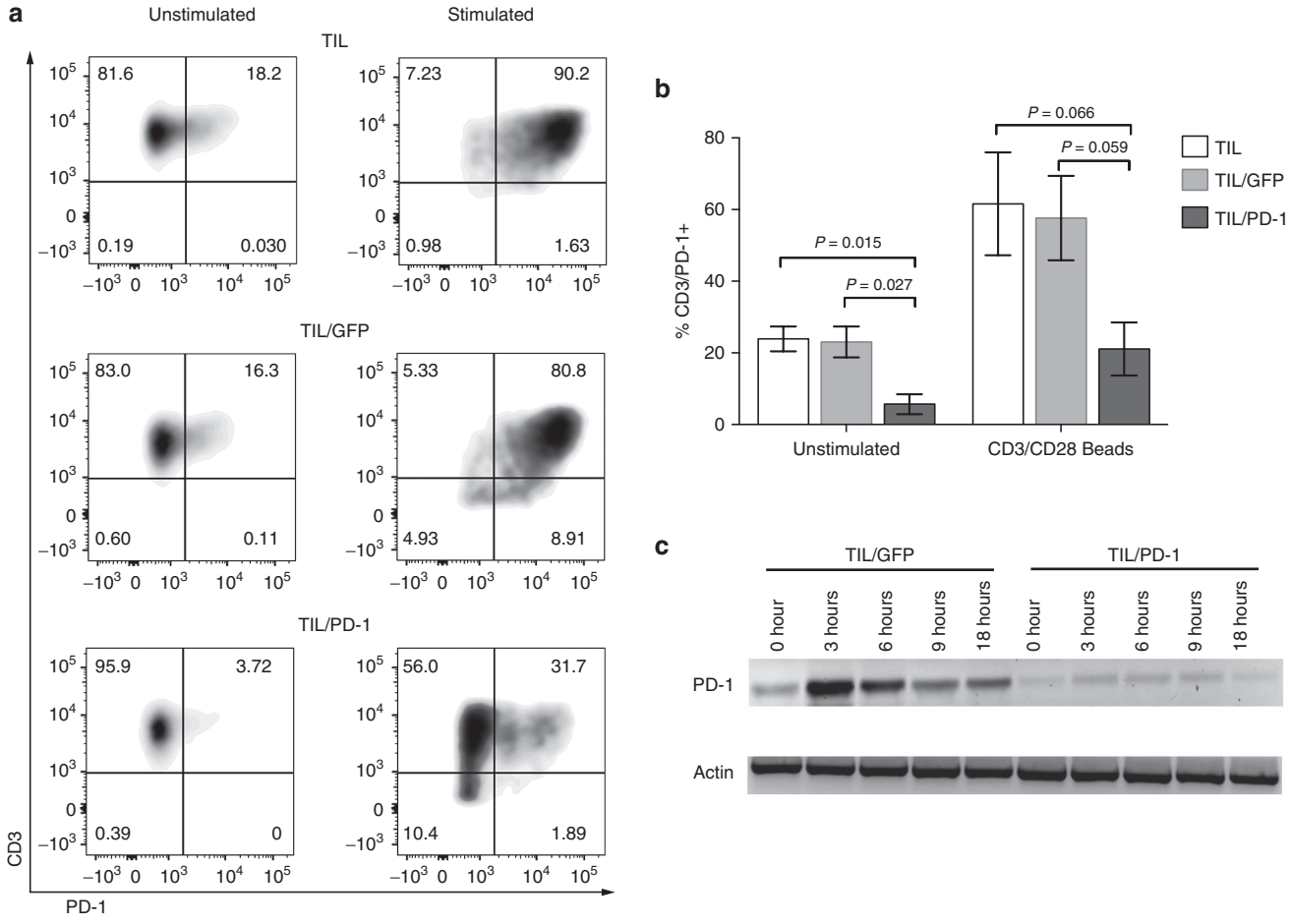

Figure 3 Expression of PD-1 is reduced following zinc finger nuclease-mediated gene editing. (**a**) PD-1 cell surface expression by FACs analysis is shown for Donor 2. Results are representative of all three donors. (**b**) Cell surface expression of PD-1 is reduced at baseline and following anti-CD3/CD28 bead stimulation in tumor infiltrating lymphocytes (TIL) following PD-1-targeted gene editing (TIL/PD-1) compared to untransfected TIL (TIL) and GFP-transfected TIL (TIL/GFP). Percent CD3⁺/PD-1⁺ cells for each condition is presented as the mean ± SEM for all three donors. (**c**) Time course of PD-1 mRNA expression in GFP transfected (TIL/GFP) and gene edited TIL (TIL/PD-1 KO) for Donor 2 during anti-CD3/CD28 bead stimulation analyzed by RT-PCR.

study were isolated from patients as previously described[25] and included both HLA-unmatched (HLA-A2$^-$; mel888 and mel938) and HLA-matched (HLA-A2$^+$; mel526, mel624, and mel624/PD-L1) targets that express the melanoma-associated antigen MART-1. TILs from Donors 1 and 2 showed recognition of the shared melanoma antigen, MART-1. PD-1 gene-edited TIL showed a significantly improved polyfunctional cytokine profile, relative to TIL/GFP controls, with release of TNFα, GM-CSF, and IFNγ ($P < 0.0001$) when cocultured with mel526. The same was true when cocultured with mel624, with the exception of IFNγ release for Donor 2, which no significant difference was observed. To further characterize the role of the PD-1/PD-L1 axis in TIL effector function, a mel624 tumor cell line was engineered to overexpress PD-L1 (mel624/PD-L1; **Supplementary Figure S3**) and was used in coculture. The increased expression of PD-L1 in the tumor cell line mel624/PD-L1 decreased the cytokine levels to those comparable with HLA-unmatched tumor cell lines in unmodified TIL from Donor 1. Importantly, while TIL/PD-1 KO cells also exhibited reduced cytokine secretion when cocultured with the mel624/PD-L1, the reduction was to a much lesser extent and significantly higher than unmodified TIL (**Figure 6**). TIL/PD-1 KO from Donor 2 also showed significantly enhanced cytokine secretion levels with the exception of IFNγ levels following coculture with mel624 and mel624/PD-L1. Donor 3, recognizing a known mutated antigen expressed by an autologous tumor cell line generated from the patient's tumor with no recognition of MART-1. TIL/PD-1 KO cells from Donor 3 showed no increase in cytokine secretion following coculture with the autologous tumor cell line (data not shown).[26] From these results, we conclude that gene editing of PD-1 in TIL significantly enhanced T-cell effector function *in vitro* as measured by the ability to secrete TNFα, GM-CSF, and IFNγ following stimulation with antigen-specific tumor targets even when the targets overexpressed PD-L1. Of note, while increased IFNγ secretion was observed for all PD-1 gene-edited TIL following coculture with mel624/PD-L1, no additional increase in cytokine secretion was observed when the

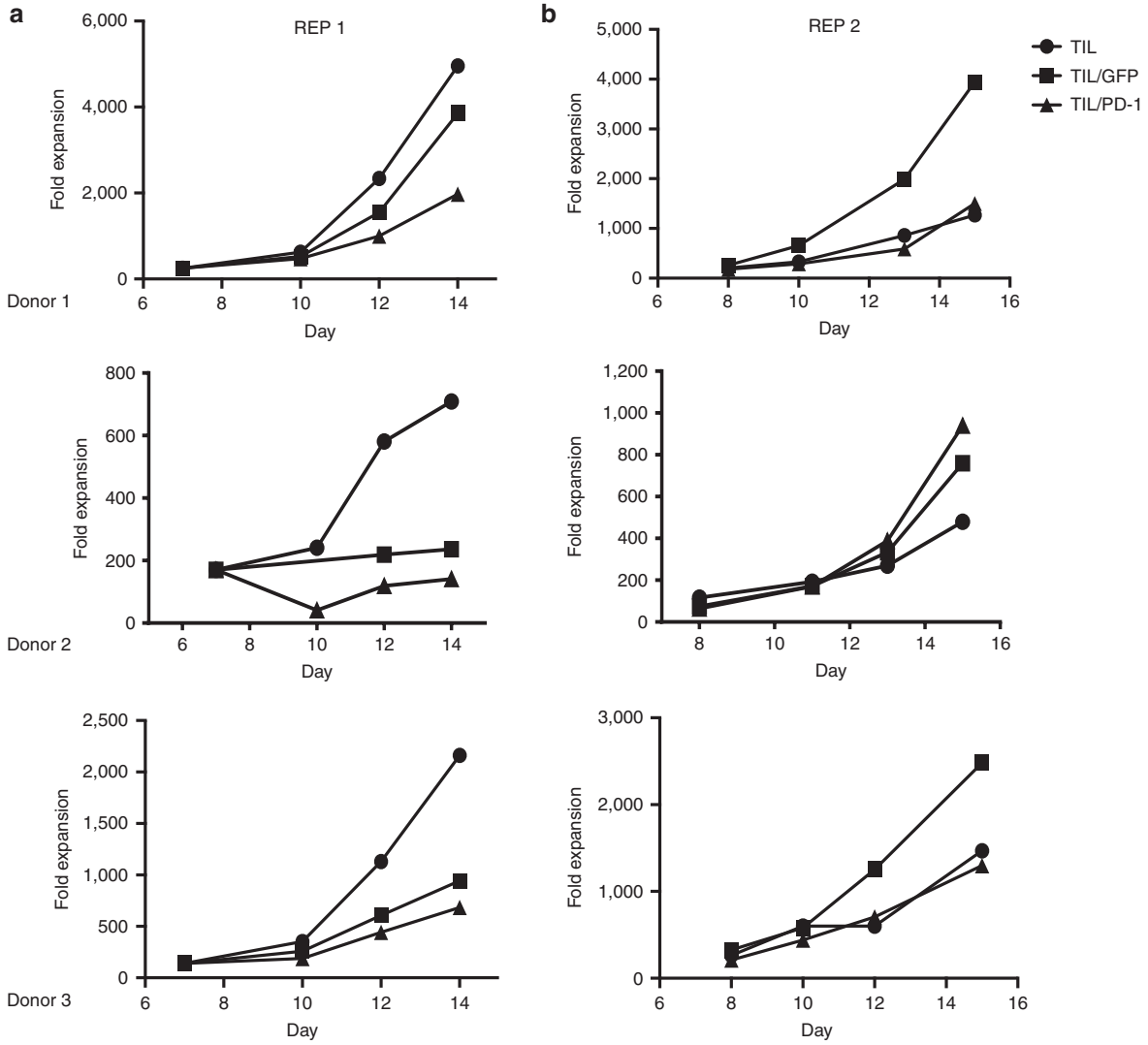

Figure 4 The effect of the flow electroporation protocol and PD-1 gene editing on tumor infiltrating lymphocytes (TIL) proliferation during rapid expansion protocol (REP). (**a**) Both GFP-transfected (TIL/GFP) and gene-edited TIL (TIL/PD-1) sustained a reduction in fold proliferation when rapid expansion was interrupted by the electroporation process (day 7). (**b**) A second REP revealed no consistent difference between TIL, TIL/GFP, and TIL/PD-1 KO with respect to fold expansion/proliferative capacity.

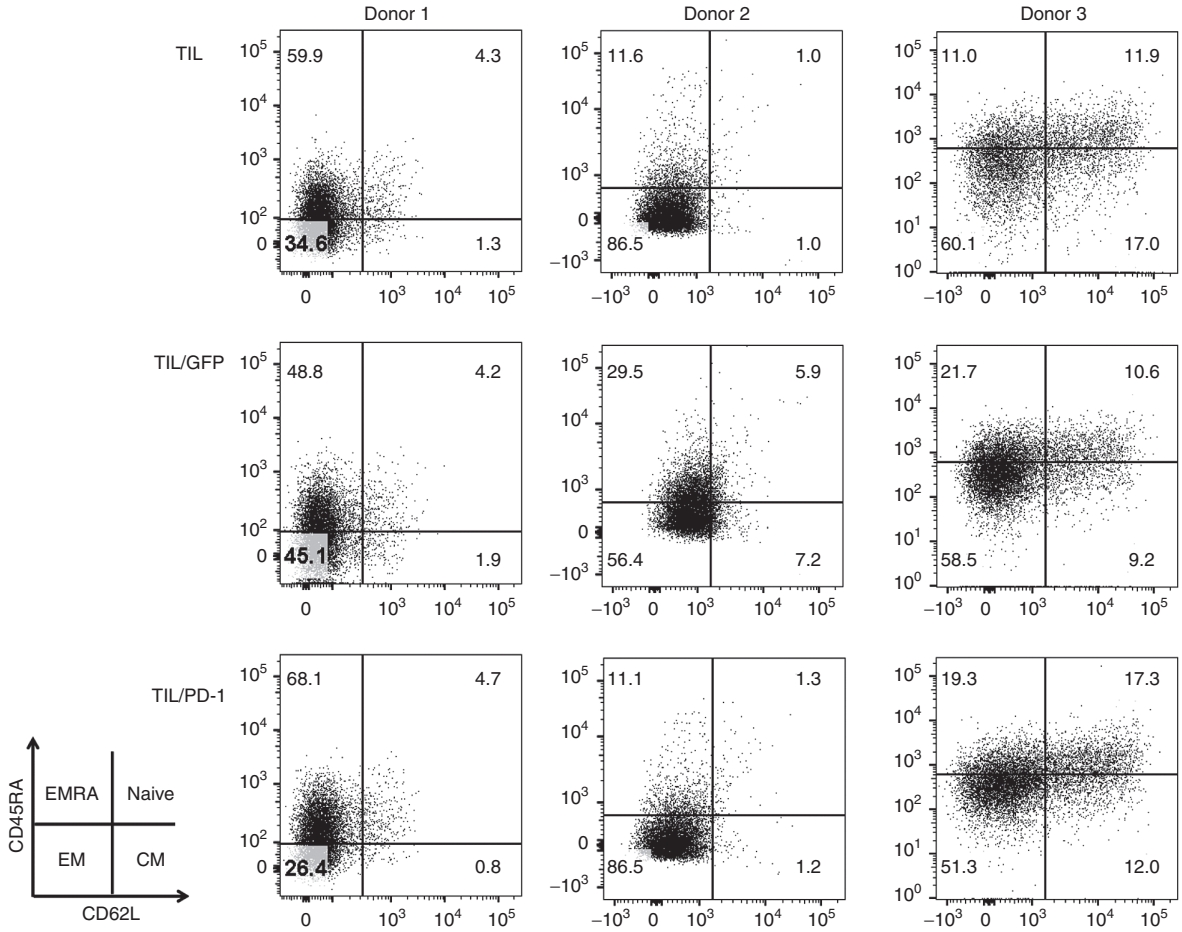

Figure 5 Gene edited tumor infiltrating lymphocytes (TIL) display a similar phenotype as unmodified cells and GFP-transfected TIL. FACs analysis was performed of each condition for all three donors. The majority of T cells within the bulk TIL had an effector memory-like phenotype ($CD45RA^-/CD62L^-$) with slight differences in number of cells expressing higher levels of CD45RA that were independent of gene editing.

PD-1/PD-L1 axis was targeted with a blocking anti-PD-1 antibody (**Supplementary Figure S2**).

To further test the effector function of ZFN treated TIL, specific cytolytic activity was measured by chromium release assay (**Supplementary Figure S4**) with cocultures of mel624 and mel624/PD-L1. PD-1 ZFN gene edited TIL from Donor 1 showed a modest improvement in target-specific cytolysis against mel624/PD-L1 as compared to untransfected TIL or TIL/GFP. For Donors 2 and 3, there were no significant differences observed between the three TIL preparations (TIL, TIL/GFP, TIL/PD-1).

Target specificity of PD-1 ZFNs

The consensus PD-1 ZFN binding sites were determined by SELEX as previously described[20,27,28] revealing highly specific binding by both ZFN monomers. To identify candidate off target cleavage sites, the SELEX determined consensus-binding sites were scanned against the human genome and potential heterodimer cleavage targets were scored according to their degree of match to the SELEX-derived base frequency matrices (**Supplementary Tables S1 and S2**). Sequencing of the 20 top-scoring potential cleavage sites was performed on both TIL/GFP and TIL/PD-1 KO from each donor and the % indel at these sites are shown in **Table 2**. Only two of the top twenty SELEX based potential off-target sites are in exonic regions. RFX2, is a gene in the regulatory factor X gene family, and with a mean rate of modification of 1.75% indels (0.82–2.30), was the only exonic target to show consistent, but relatively modest gene modification. In contrast, the mean on target PD1 gene modification was 75% ($P = 0.004$).

In vivo toxicity studies in NSG mice

PD-1 gene-edited and mock transfected TIL were transferred into NSG mice to evaluate whether *ex vivo* knockout of PD-1 in T lymphocytes may result in proliferative abnormalities or tumor formation. In this study, 5 million TIL/PD-1 or TIL/GFP from three separate donors were transferred into NSG mice (five mice per group, **Table 3**) without IL-2 supplementation. Engraftment was assessed post-cell transfer by measuring the frequency of human $CD3^+/CD45^+$ cells circulating in the peripheral blood. Low-level engraftment was detected 1-week postinfusion (0.06–3.44%), with no observable difference between the GFP transfected or PD-1 gene-edited TIL. Engraftment of TIL at 1 month post-cell transfer reduced to near background levels, at which point the experiment was terminated and euthanized mice were evaluated at necropsy. No tissue abnormalities or tumor formation was observed following H&E staining of formalin-fixed tissues (liver, lung, spleen, thymus, and bone marrow). Based on the lack of

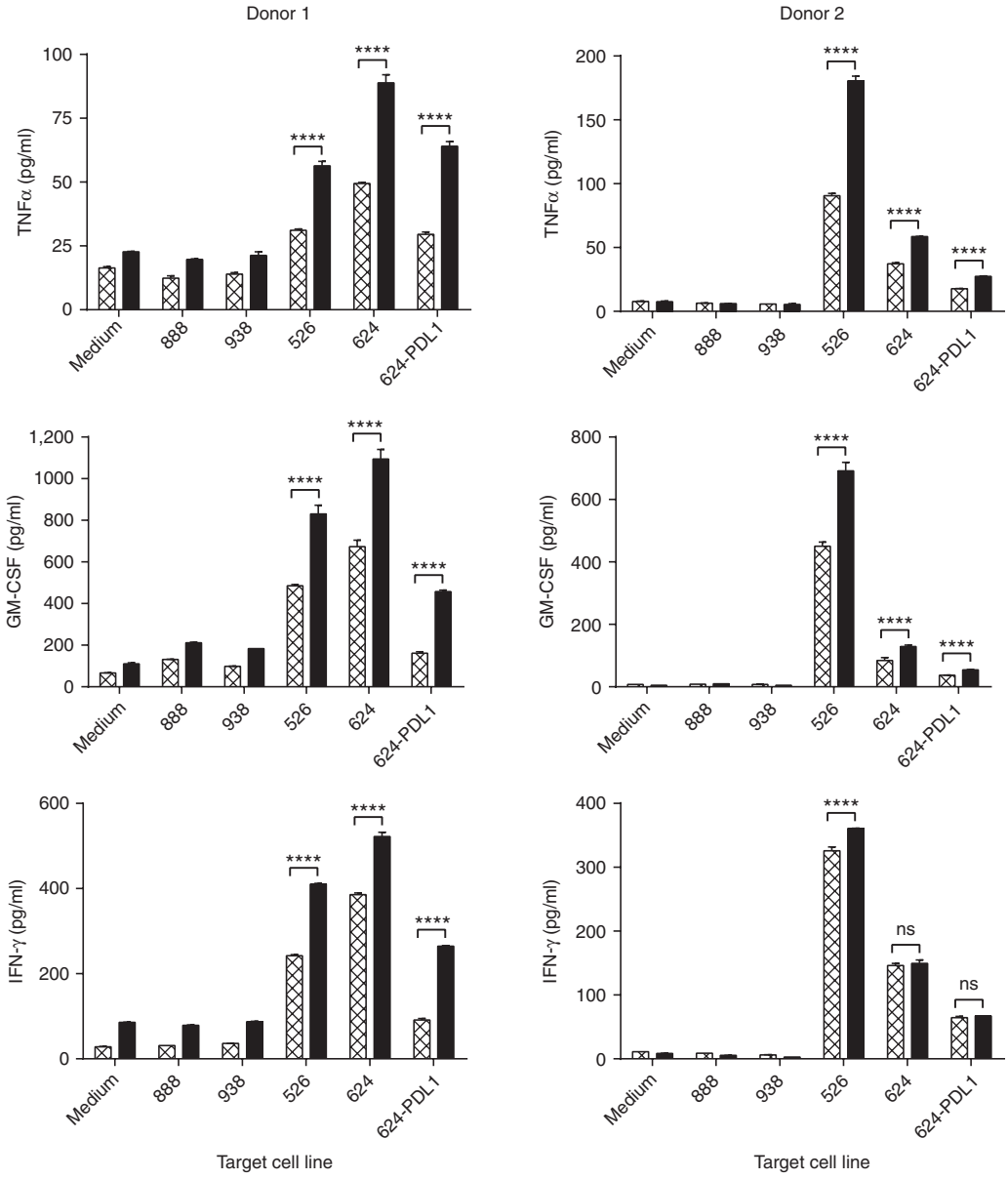

Figure 6 Enhanced polyfunctional effector cytokine profile of PD-1 knockout tumor infiltrating lymphocytes (TIL) *in vitro*. Coculture with melanoma cell lines was performed on day 12 of the rapid expansion protocol, 5 days after electroporation, and cytokine (CXCL8/IL-8, GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, TNFα, and VEGF) levels were measured using the Luminex Performance Human High Sensitivity Cytokine Panel. Donor 1 showed a polyfunctional effector phenotype and a significant increase in TNFα, GM-CSF, and IFNγ secretion following PD-1 gene editing (black bars) as compared to the unmodified TIL ($P < 0.0001$, hatched bars). Donor 1 PD-1 KO TIL also showed significantly less inhibition following coculture with mel624/PD-L1, a PD-L1-overexpressing cell line. Similar results seen for Donor 2; however, no significant difference in IFNγ secretion was observed when compared to unmodified TIL with the exception of Donor 2 PD-1 KO TIL cocultured with mel526. Shown are representative data from Donors 1 and 2, one-way analysis of variance, $P \leq 0.0001$).

long-term circulating human T cells in the periphery and no evidence of tissue abnormalities, PD-1-ZFN-related carcinogenicity was not observed (**Table 3**).

DISCUSSION

With a response rate of 50%, adoptive cell transfer (ACT) using autologous TIL offers an effective therapy for metastatic melanoma.[5] Building on this success, efforts are ongoing in to further improve response rates by developing better methods to manufacture TIL, better selection of tumor reactive T cells within the bulk TIL population,[15,29] and by preventing the downregulation of T-cell effector functions mediated by the immunosuppressive tumor microenvironment. To that end, we exploited a gene editing technology, which involves the disruption of the human PD-1 gene locus using ZFNs. Herein, we demonstrate that delivery of ZFNs via a high flow-through electroporation system provides an efficient platform to decrease PD-1 expression in TIL at a scale and efficiency suitable for patient treatment. Our results indicate that gene modification was highly efficient (70–84% disruption at the PD-1 locus) and resulted in a significant decrease in both PD-1 mRNA and cell surface expression on treated T cells. We demonstrate how a pair of ZFNs directed against exon 1 of the PD-1 gene can result

Table 2 PD-1 gene editing by ZFNs is target specific

Off-target site				% Indels, mean (SEM)[b]			
				GFP-Mock		PD1-KO-Large	
Ranking[a]	Chromosome	Gene	Exon/intron	Measured	Background corrected	Measured	Background corrected
1	Chr2	PDCD1	Intron	0.27 (0.17)	0	74.83 (4.68)	74.56
2	Chr15	Intergenic	Intergenic	0.09 (0.01)	0	0.20 (0.08)	0.11
3	Chr13	PHF2P1	Intron	0.16 (0.04)	0	0.17 (0.02)	0.01
4	Chr10	C10orf72	Intron	0.04 (0.02)	0	0.06 (0.02)	0.02
5	Chr21	Intergenic	Intergenic	0.21 (0.02)	0	0.14 (0.02)	0
6	Chr7	Intergenic	Intergenic	0.19 (0.02)	0	0.19 (0.05)	0
7	Chr21	Intergenic	Intergenic	0.03 (0.01)	0	0.52 (0.17)	0.49
8	Chr11	Intergenic	Intergenic	0.08 (0.02)	0	0.08 (0.01)	0
9	Chr19	TTYH1	Intron	0.56 (0.22)	0	0.72 (0.09)	0.16
10	Chr13	MCF2L	Intron	0.66 (0.11)	0	0.48 (0.10)	0
11	Chr3	CADM2	Intron	0.07 (0)	0	0.09 (0.02)	0.02
12	Chr8	KCNQ3	Intron	0.13 (0.03)	0	0.38 (0.14)	0.25
13	Chr19	HNRNPUL1	Intron	0.26 (0.06)	0	0.24 (0.04)	0
14	Chr19	RFX2[c]	Exon	0.04 (0.01)	0	1.78 (0.47)	1.74
15	Chr17	Intergenic	Intergenic	0.73 (0.39)	0	0.78 (0.43)	0.05
16	Chr10	Intergenic	Intergenic	0.08 (0.01)	0	0.07 (0.02)	0
17	Chr4	SORCS2	Intron	0.27 (0.05)	0	0.28 (0.03)	0.01
18	Chr20	NFATC2	Intron	1.48 (0.15)	0	1.47 (0.10)	0
19	Chr19	U2AF2	Exon	0.47 (0.08)	0	0.64 (0.16)	0.17
20	Chr13	PHF2P1	Intron	0.21 (0.03)	0	0.55 (0.11)	0.34
21	Chr12	C12orf34	Intron	0.15 (0.01)	0	0.20 (0.02)	0.05

[a]The SELEX predicted top ranked target site (first row, ranking = 1) is the PDCD1 gene, with a mean gene modification of ~75%. The next top 20 SELEX predicted potential cleavage sites are shown here and in **Supplementary Table S2**. [b]Deep sequencing analyses were performed on both TIL/GFP and TIL/PD-1 KO from each donor. The mean % indels and standard deviation (in parenthesis) at these sites for all donors are shown. [c]RFX2 (ranking #14) was the only exonic gene locus to show consistent gene modification in all three donors, with a modest mean indel of 1.75% (range = 0.82–2.30).
TIL, tumor infiltrating lymphocytes.

Table 3 *In-vivo* toxicity assessment of engrafted PD-1 knockout melanoma TIL

Donor	Group	N	Cell engrafment at day 7 post-transfer[a]	Cell engrafment at day 30 post-transfer[a]	Unscheduled deaths[b]	% GVHD[c]	Pathology findings[d]
1	PD-1 ZFN	5	1.54 ± 0.050	0.03 ± 0.01	0/5	0/5	0/5
	Mock	5	3.44 ± 3.20	0.04 ± 0.01	0/5	0/5	0/5
2	PD-1 ZFN	5	0.06 ± 0.03	0.15 ± 0.01	0/5	0/5	0/5
	Mock	5	0.07 ± 0.00	0.26 ± 0.11	0/5	0/5	0/5
3	PD-1 ZFN	5	1.02 ± 1.80	0.12 ± 0.02	0/5	0/5	0/5
	Mock	5	0.48 ± 0.07	0.13 ± 0.02	0/5	0/5	0/5

[a]Engraftment of transferred human TIL was based on the frequency of human CD3+/CD45+ T cells in the peripheral blood. [b]One of five mice in the Mock control group for donor 2 died. Death was not attributed to xGVHD and not associated with the transferred cells. [c]xGVHD was determined by more than one observation of loss of activity, ruffled fur, change in posture or body weight. [d]Study terminated on day 30 post-cell transfer. Pathological findings following necropsy on review of H&E stained formalin-fixed sections of the liver, lung, spleen, and thymus. In addition, bone marrow (BM) was similarly assessed following Giemsa-Wright staining of BM smears.
GVHD, graft versus host disease; TIL, tumor infiltrating lymphocytes.

in bi-allelic disruption of the gene locus with high efficiency, *i.e.*, approaching 50% of the TIL population without selection for the desired effect. While there was a decrease in the cell expansion during the initial electroporation/REP cycle (seen in both the TIL/GFP and TIL/PD-1 samples), the difference was transient and the T cells retained a proliferative capacity similar to untransfected TIL following a second REP. The TIL (mock or PD-1 KO) were not adversely affected by the electroporation process and were mostly of the effector memory phenotype. TIL/PD-1 KO exhibited a polyfunctional cytokine secretion profile and released significantly higher levels of TNFα, GM-CSF, and IFNγ for donors 1 and 2, with the exception of IFNγ for donor 2, in an antigen-specific manner

as compared to TIL/GFP. In addition, TIL/PD-1 maintained their capacity to secrete significantly higher levels of cytokine when cocultured with a tumor cell line that overexpresses PD-1 inhibitory ligand, mel624/PD-L1. Taken together, these results suggest that ZFNs can be used to create PD-1-deficient TIL with significantly enhanced effector function at a scale necessary for clinical study in subjects with metastatic melanoma.

For our preclinical study, it was important to determine if a reduction in PD-1 expression on TIL was important for tumor-specific effector function or target-mediated cell lysis. An important function of PD-1 is the decrease in functional capacity when its ligand, PD-L1, is engaged.[30] As such, PD-1 blockade should release the cells from PD-L1-induced suppression. While some have shown that blocking the PD-1/PD-L1 axis *in vitro* and in some murine models resulted in increased *in vitro* effector function (increased IFNγ secretion or cytolysis),[31] this is not a general finding. Our findings showed a significant increase in *in vitro* effector function based on a polyfunctional cytokine profile following coculture with antigen-specific target cells. Comparison of PD-1 gene knockout versus human anti-PD-1 monclonal antibody blockade was attempted, but our efforts to demonstrate a positive effect on TIL effector function following PD-1 blockade were inconclusive (**Supplementary Figure S2**).[32] Data to support the complete blockade of the PD-1/PD-L1 axis using the anti-PD-1 monoclonal antibody (clone EH12.2H7) are lacking. In addition, only modest benefits in cell proliferation have been reported following PD-1 blockade on T lymphocytes, which does not necessarily directly translate into a more tumor-reactive population of TIL.[33,34]

There is a growing body of evidence suggesting that TIL cause tumor regression through the recognition of mutated antigens, making it even more difficult to extrapolate murine tumor models or humanized mouse models to the human TIL setting.[29,35] The most compelling evidence for the role of the PD-1/PD-L1 axis in evading the host immune response to cancer comes from a growing body of clinical trials where blocking the PD-1/PD-L1 interaction with a monoclonal antibody has been shown to induce tumor regression in patients with metastatic melanoma, renal cell, colonic adenocarcinoma, and non–small-cell lung cancer.[13,36,37] Treatment with an anti-PD-1 antibody can result in an objective response as high as 38% in patients with metastatic melanoma.[13] However, there are adverse events related to inflammation and autoimmunity with the systemic therapy that include pneumonitis, renal dysfunction, and elevation in aminotransferase levels.[12] Our proposed strategy to silence PD-1 *ex vivo* in TIL prior to adoptive cell transfer could confer the advantages of blocking the PD-1/PD-L1 axis while avoiding the toxicities associated with the administration of a systemic antibody.

The heterodimer-dependent cleavage by the Fok-I nuclease domain of the ZFN mandates binding of both ZFPs to a targeted region prior to DNA cleavage. As part of our preclinical evaluation, we performed off target analysis by sequencing the top 20 SELEX predicted potential cleavage sites from ZFN-treated cells. Only two of these sites were in an exonic region. RFX2 was the only exonic target to show consistent, but relatively modest gene modification, with a measured rate of modification of 1.75% indels. RFX2 is a transcriptional activator and can bind to cis elements in the promoter of the IL-5 receptor alpha gene, yet the full biological role remains unknown.[38] Based on the previously established safety profile of ZFNs, the minimal off-target gene modification in our analysis, continued *in vitro* dependence on IL-2 following electroporation (**Supplementary Figure S5**), no proliferative abnormalities and no observed tumorgenicity in an NSG engraftment model (**Table 3**), this platform appears safe for further evaluation.

The implications of PD-1 gene editing may reach beyond the current application of TIL for the treatment of metastatic melanoma. We have previously shown that PD-1 expression on CD8+ TIL accurately identifies the repertoire of clonally expanded tumor-reactive cells.[15] Adoptively transferred T cells, enriched by sorting for tumor-reactive markers like PD-1, may benefit from gene editing of the PD-1 locus prior to ACT. This would effectively create a tumor-specific population of T cells less susceptible to the immunosuppressive effects of tumor PD-L1 expression. Another application is that of T-cell receptor (TCR) or chimeric antigen receptor (CAR)-engineered lymphocytes which can induce cancer regression in patients not amenable to TIL therapy.[39–43] Using expression profiling, we have previously reported the overexpression of multiple inhibitory receptors, including PD-1, in TCR-engineered lymphocytes that persist *in vivo* following ACT.[44] Our present study validates the use of a platform that could be used for gene editing of PD-1 in TCR or CAR-transduced T cells prior to ACT.

In summary, ZFNs provide an efficient way to knock out PD-1 expression in TIL at clinical scale without adversely affecting T-cell effector function or altering the TIL phenotype. Gene modification is specific while supporting on-target cleavage levels that result in bi-allelic disruption of PD-1 in approximately half of the TIL population. Using this approach to improve the efficacy of TIL and other T-cell-based therapies in patients with cancer warrant further clinical investigation.

MATERIALS AND METHODS

PD-1 ZFN construct and ZFN messenger RNA. ZFNs targeting the human PDCD1 (PD-1) gene were designed by modular assembly using an archive of one- and two-finger modules and optimized for binding as previously described (**Figure 1a**).[45] DNA segments encoding the two ZFNs were then linked into a single gene that expresses both ZFNs separated by a 2A self-processing polypeptide, and the resulting gene was subsequently cloned into a T7 expression cassette (Invitrogen, Carlsbad, CA) for *in vitro* transcription of ZFN encoding mRNA. mRNA was made using a standard *in vitro* transcription method by a commercial vendor (Trilink Biotechnologies, San Diego, CA). The full amino acid sequence of the PD-1 ZFNs (PD1-ZFN-L and PD1-ZFN-R) is disclosed in the supporting supplementary information (**Supplementary Figure S1**).

Cell culture and cell lines. All human cells and tissues were obtained under Institutional Review Board approved protocols (National Cancer Institute, Bethesda, MD). Melanoma tumor cell lines HLA-A2$^+$/MART-1$^+$ (526, 624, 624/PD-L1) and HLA-A2$^-$/MART-1$^+$ (888, 938) were generated at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD), as previously described.[25] Adherent tumor cell lines were grown under standard conditions in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum medium (Sigma Aldrich, St. Louis, MO), 25 mmol/l HEPES (Invitrogen) and penicillin/streptomycin (Invitrogen) at 37 °C with 5% CO_2. TIL were cultured in G-Rex100 flasks (Wilson-Wolf Manufacturing, New Brighton, MN) with T-cell medium (AIM V, Invitrogen) plus 5% human AB serum, 25 mmol/l HEPES, penicillin/streptomycin, and 500 IU/ml interleukin-2 (IL-2, Aldesleukin, Prometheus, San Diego, CA).

REP and TIL electroporation. As shown in **Figure 1b**, TIL electroporation was performed using the MaxCyte GT Flow Transfection System (MaxCyte, Gaithersburg, MD). In order to generate a sufficient number of transduced T cells for adoptive cell transfer, the TIL were induced to proliferate using a REP.[46] Briefly, 1×10^7 TIL were combined with 1×10^9 allogeneic, irradiated (5,000 rad) peripheral blood mononuclear cells (PBMC), and these cells were suspended in 400 ml of T-cell media containing 30 ng/ml of OKT3. The cells were cultured in a G-Rex100 flask at 37 °C and 5% CO_2. Five days later, 200 ml of media was aspirated and replaced. Seven days after the start of the REP, TIL were harvested and washed two times with Hyclone Electroporation Buffer (Hyclone Laboratories, Logan, UT). Cells were then counted and resuspended in electroporation buffer at a concentration of 1×10^8/ml. Cells were then transferred to the MaxCyte CL-2 processing assembly and mixed with 120 μg/ml of PD-1 ZFN mRNA (or GFP mRNA for GFP transfected TIL/GFP). Electroporation was performed as per MaxCyte's protocol. Following electroporation, TIL were transferred from the processing assembly to a T-175 flask and placed in an incubator at 37 °C for 20 minutes. Following this incubation step, TIL were resuspended in AIM-V media at a concentration of 1×10^6/ml. Cells were then placed in an incubator set at 30 °C for an overnight low temperature incubation as previously described.[47] The following day, TIL were transferred to a 37 °C incubator and left undisturbed until REP day 10 (3 days following electroporation).

Cel-1 and RT-PCR assay. The level of ZFN-induced PD-1 gene modification was quantified by the Cel-1 assay as previously described.[48] Cel-1 assay was performed using the forward primer 5′- ACCTGGGACAGTTTCCCTTC and the reverse primer 5′- CTGGCTCTGGGACACCTG to target PD-1 exon 1 region.

To evaluate the expression timecourse of PD-1 mRNA, TILs were electroporated with either GFP mRNA or PD-1 ZFN mRNA. Following electroporation, cells were restimulated with anti-CD3/CD28 beads. Transduced cells were then harvested at 0, 3, 6, 9, and 18 hours following stimulation after which total RNA was isolated from treated cells. RT-PCR assay was performed with 1 μg total RNA using SuperScript III (Life Tech, CA) along with the human PD-1 forward primer, ACCTGGGACAGTTTCCCTTC, and reverse primer, CTGGCTCTGGGACACCTG. For PCR control reactions, human β-actin was amplified at the same time with forward, ACACTGTGCCCATCTACGAGG, and reverse, AGGGGCCGGACTC GTCATACT, primers, respectively.

Generation of gene-edited TIL clones. Using a modified FACS Aria instrument (BD Biosciences, San Jose, CA), single cells were sorted into individual wells of a 96-well plate containing 100 allogeneic, irradiated (5,000 rad) PBMC, in 150 μl of T-cell media with 30 ng/ml of OKT3. Plates were covered with aluminum foil to prevent desiccation and incubated at 37 °C with 5% CO_2. Seven days later, 100 μl of T-cell media (containing OKT3) was added to each well and plates were returned to the incubator. On day 14 of the culture, the T-cell clones were harvested and genomic DNA was isolated for clonal assessment of PD-1 gene modification.

Indel quantification via next-generation sequencing/MiSeq. Loci of interest (both on target PD1 as well as potential off-target sites) were PCR amplified, and the levels of modification at each locus were determined by paired-end deep sequencing on an Illumina MiSeq sequencer. Paired sequences were merged via SeqPrep (John St. John, https://github.com/jstjohn/SeqPrep, unpublished). A Needleman-Wunsch alignment was performed between the target amplicon genome region and the obtained Illumina sequence to map indels.[49]

Flow cytometry and PD-1 induction. FACS was performed using an APC conjugated anti-PD1 antibody (eBioscience, San Diego, CA, clone MIH-4) according to manufacturer's recommendations. FACS analysis was performed on day 14 of the REP, 7 days following electroporation. To induce PD-1 expression, TIL were activated with anti-CD3/CD28 Dynabeads (Life Technologies, Grand Island, NY). Cells were plated in a 24-well tissue culture plate at 1×10^6/ml at a ratio of four beads/cell for 48 hours prior to FACS analysis. Cells were analyzed using a FACScan flow cytometer with FlowJo software (Tree Star, Ashland, OR).

Coculture and cytokine assays. 1×10^5 TIL were combined with an equal number of target cells in the indicated well of a 96-well plate containing T-cell media without IL-2. The cells were incubated at 37 °C overnight. The following day, the supernatant from each well was collected and an IFNγ ELISA assay (Pierce, Rockford, IL) was performed according to manufacturer's protocol. In addition, secreted cytokine (CXCL8/IL-8, GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, TNFα, and vascular endothelial growth factor (VEGF)) levels were also measured using the Luminex Performance Human High Sensitivity Cytokine Panel (R&D systems, Minneapolis, MN) according to manufacturer's protocol. Cocultures were also performed using a PD-1 blocking antibody as a treatment condition for comparison (**Supplementary Figure S2**). For these experiments, 1×10^5 TIL were incubated for 1 hour at 37 °C in T-cell media without IL-2 along with either 5 or 30 μg/ml of Ultra-LEAF (low endotoxin, azide-free) purified anti-human PD-1 blocking antibody (clone EH12.2h7, BioLegend, San Diego, CA) prior to plating an equal number of target tumor cells.

Cytolytic assay. Melanoma tumor cell lines were incubated for 120 minutes with 100 μCi chromium 51 (^{51}Cr) (PerkinElmer, Boston, MA) and washed three times with culture media. Chromium-labeled melanoma cell lines were plated at 1×10^4 cells/well of a 96-well U-bottom plate along with either TIL, TIL/GFP, or TIL/PD-1 KO cells at the indicated effector/target ratios. The coculture was incubated for 4.5 hours at 37 °C. The supernatant was harvested, and the amount of ^{51}Cr released was determined by γ-counting. Tumor specific lysis was calculated from triplicate samples using the following equation ((experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm)) and expressed as a percentage.[25]

Off-target analysis. Binding sites recognized by PD1-L and PD1-R were determined using a SELEX approach as described.[20] This study yielded the base frequency matrices summarized in **Supplementary Table S1**. The human genome (hg19) was then scanned for potential heterodimer cleavage targets using the approximate string-matching algorithm and core consensus sequences derived from **Supplementary Table S1** (GTCGTYMGGGYGGTG for PD1-ZFN-R and AGGGCGGCTGTGGGA for PD1-ZFN-L).[27] Candidate targets were then scored and ranked using full base frequency matrices essentially as described to yield the ranked list provided in **Supplementary Table S2**.[28] For identifying and ranking candidate cleavage sites, a separation distance of 5 or 6 bp was allowed between ZFN binding sites, reflecting the ability of ZFNs to efficiently dimerize and cleave in either configuration.

In vivo *toxicity studies in NSG mice.* Four- to 6-week-old NOD. Cg-Prkdc^{scid}Il2rg$^{tm1Wj1/SzJ}$ (NSG) female mice (the Jackon Laboratory, Bar Harbor, ME) were used for adoptive cell transfer experiments. All procedures were approved by the National Cancer Institute animal care and use committee. Briefly, each mouse received an intravenous injection of $1–5\times10^6$ unmodified or PD-1 KO TIL resuspend in 500 μl of HBSS. Peripheral blood was collected at 1 week and 1 month post-cell transfer and stained for CD3, CD4, CD8, and CD45. Daily cage observations were made to assess gross animal health and signs of xGVHD. Following termination of the experiments, mice were subjected gross visual assessment at necropsy. In addition, multiple organs (spleen, liver, lung, thymus, bone marrow, bone) were collected. Samples were split for DNA isolation (snap freeze) and formalin fixation followed by sectioning and H & E staining and subsequent analysis. Any gross pathological abnormalities were analyzed further by immunohistochemical staining for T cell infiltration.

Statistical analysis. Continuous variables were expressed as median with range or mean ± standard error of mean or standard deviation and compared using analysis of variance (ANOVA). For significant differences, a *post hoc* analysis was performed using the Student's *t*-test for 2×2

comparison. Statistical significance was set at $P \leq 0.05$. Analysis was performed using Graphpad Prism software v6.0b (La Jolla, CA).

SUPPLEMENTARY MATERIAL

Figure S1. Amino acid sequence of PD1-ZFN-L and PD1-ZFN-R.
Figure S2. IFNγ secretion by TIL when cocultured with mel624/PD-L1 (donors 1 and 2) or an autologous tumor cell line (donor 3).
Figure S3. PDL-1 expression by FACs analysis on target melanoma tumor cells lines: HLA-A2–/MART-1+ (888, 938) and HLA-A2+/MART-1+ (526, 624/BSR, 624/PDL-1).
Figure S4. PD-1 knockout TIL retain lytic potential in vitro.
Figure S5. Growth curves of each TIL preparation (TIL, TIL/GFP, TIL/PD-1) for donors 2 and 3 following IL-2 withdrawal from culture media.
Table S1. Base frequency matrices for PD1-L and PD1-R, as derived from an alignment of sequences recovered via SELEX analysis.
Table S2. Top ranking candidate PD1 ZFN cleavage sites in the human genome.

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